Esp

Week 3

Summarized below are the experiments conducted this week in chronological order. Click on the experiment name to view it. To go back to this summary, click **Summary** in the footer.

Summary

1 Digestion of EB, T7 plasmid and PCR product

Responsible

Aman Mebrahtu and Bethel Tesfai Embaie

Protocols used

- 3A Assembly, subsection 'Digestion'

Experimental Set-up

For each reaction, 100 ng of DNA were digested:

Table 1: Samples and reactions performed

Sample	Sample Concentration [μl]	Reaction
EB Plasmid	148.8	sd EcoRI (Duplicate) sd SpeI (Duplicate) dd EcorI & SpeI (Duplicate)
T7 Plasmid	100.8	sd EcoRI (Duplicate) sd SpeI (Duplicate) dd EcoRI & SpeI (Duplicate)
T7 PCR product	50	sd EcoRI (Duplicate) sd SpeI (Duplicate) dd EcoRI & SpeI (Duplicate)

Table 2: Master Mix EcoRI

Component	Volume [μl]
ddH_2O	20.4
10x cutsmart	2.4
Enzyme	1.2
Total Volume	24

Table 3: Master Mix SpeI

Component	Volume [μl]
ddH_2O	20.4
10x Cutsmart Buffer	2.4
Enzyme	1.2
Total Volume	24

Table 4: Digestion Reaction

Component	Volume [μl]
Enzyme Master Mix 1	2
Enzyme Master Mix 2 (if needed)	2
DNA Sample	corresponding to 100 ng
10x Cutsmart Buffer	0.8
ddH_2O	Fill up to 8

Results and Conclusions

Table 5: Gel Elecrophoresis

Well	Sample	Expected Size [bp]	Conclusion
1	DNA Ladder		
2 & 3	EB Plasmid sd EcorI	2901	Successful
4 & 5	EB Plasmid sd SpeI	2901	”
6 & 7	EB Plasmid dd	2047 and 854	”
8 & 9	T7 plasmid sd EcorI	2187	”
10 & 11	T7 plasmid sd SpeI	2187	Successful
12 & 13	T7 plasmid dd	2132 and 55	Likely successful*
14 & 15	T7 PCR product sd EcorI	228 and 118	Successful
16 & 17	T7 PCR product sd SpeI	173	Successful
18 & 19	T7 PCR product dd	170, 119 and 55	Successful

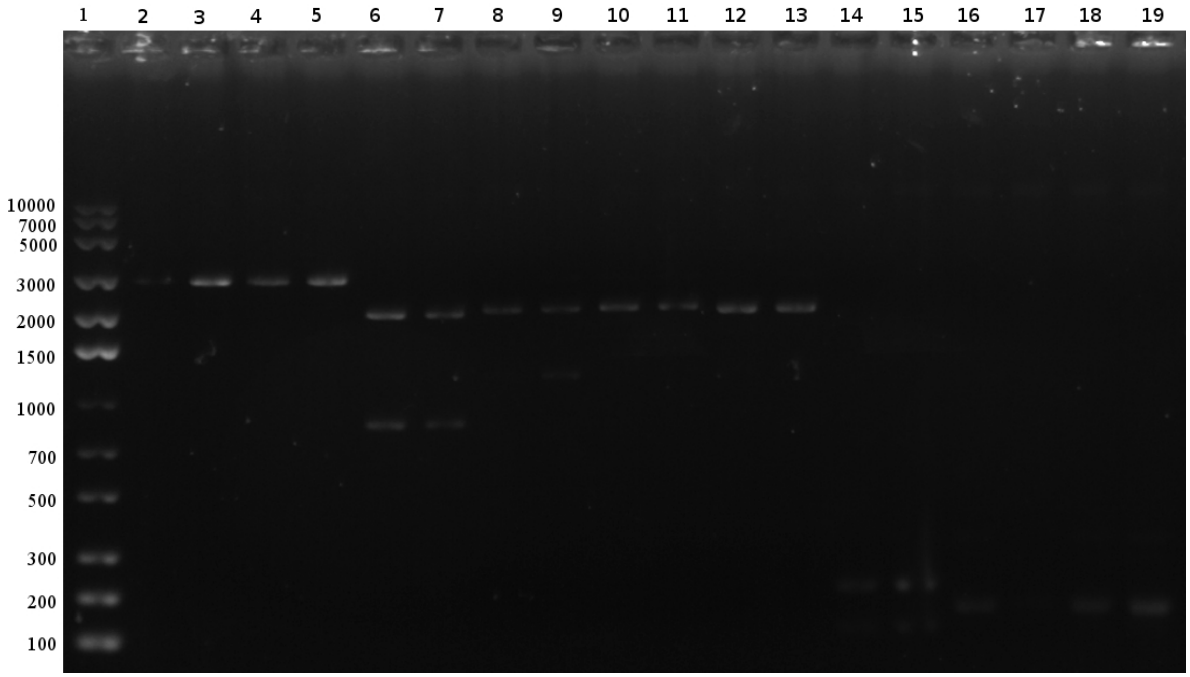

Figure 1: Analysis of digested EB and T7, demonstrating successful digestion in most cases.

*Success of T7 double digestions are hard to judge since the 55bp insert of interest is too short to be efficiently visualized on a standard gel. However, the longer fragments appear to be of the correct size so that correct digestion of T7 can be assumed even if the actual T7 fragment can not be seen.

Discussion and Troubleshooting

In this experiment we were able to confirm the functionality of our restriction enzymes. Both single and double digestions appear to largely have been successful. Thanks to performing duplicates, we were able to conclude that digestions worked as expected despite a contaminated well in the T7 plasmid digestion and a seemingly empty well for the T7 PCR product digestion.

In a next step, the digested fragments can be ligated together with an appropriate backbone.

2 Digestion of Nu, EB, EC, Ly, T7, pSB1K3

Responsible

Aman Mebrahtu, Oscar Frisell, Oscar He, Bethel Tesfai Embaie, Sigrun Stulz

Protocols used

- 3A Assembly, subsection 'Digestion'
- Gel Purification

Modifications to protocols

In order to obtain enough products from the double digest to efficiently gel purify, 3 double digestions were performed for each sample and added together into one column for purification. This applies for all samples except the T7 samples which, due to their small size, can not be PCR or gel purified with conventional methods. Since they are not digested with PstI, purification is not absolutely necessary.

Experimental Set-up

Table 6: Samples and reactions performed

Sample	Sample Concentration [μl]	Reaction
Nu	too low, no reactions performed	
EB	148.8	sd XbaI sd PstI dd XbaI & PstI (Triplicate)
EC	100	sd XbaI sd PstI dd XbaI & PstI (Triplicate)
Ly	107	sd XbaI sd PstI dd XbaI & PstI (Tripliate)
T7 PCR product	280.3	dd EcoRI & SpeI
pSB1K3	25	dd EcoRI & PstI (Triplicate)

*Triplicate should have been performed here as well but due to too little PCR product to start out with, only one double digestion was performed.

Table 7: Master Mix EcoRI

Component	Volume [μl]
ddH_2O	11.9
10x cutsmart	1.4
Enzyme	0.7
Total Volume	14

Table 8: Master Mix PstI

Component	Volume [μl]
ddH_20	25.5
10x Cutsmart Buffer	3
Enzyme	1.5
Total Volume	30

Table 9: Master Mix SpeI

Component	Volume [μl]
ddH_2O	6.8
10x Cutsmart Buffer	0.8
Enzyme	0.4
Total Volume	8

Table 10: Master Mix XbaI

Component	Volume [μl]
ddH_2O	20.4
10x Cutsmart Buffer	2.4
Enzyme	1.2
Total Volume	24

Table 11: Digestion Reaction

Component	Volume [μl]
Enzyme Master Mix 1	2
Enzyme Master Mix 2 (if needed)	2
DNA Sample	corresponding to 100 ng
10x Cutsmart Buffer	0.8
ddH_2O	Fill up to 8

Results and Conclusions

Table 12: Gel Elecrophoresis 1

Well	Sample	Expected Size [bp]	Conclusion
1	DNA Ladder		
2	EB undigested	2901	Successful
3	EB sd XbaI	2901	”
4	EB sd PstI	2901	Ran as smear, perhaps successful
5 & 6 & 7	EB dd XbaI and PstI	2044 and 857	”
8	EC undigested	2935	Successful
9	EC sd XbaI	2935	Contamination
10	EC sd PstI	2935	Ran as smear, appears bigger than expected
11 & 12	EC dd XbaI and PstI	2048 & 887	many bands, some of ŕight size
13	”	”	unsuccessful

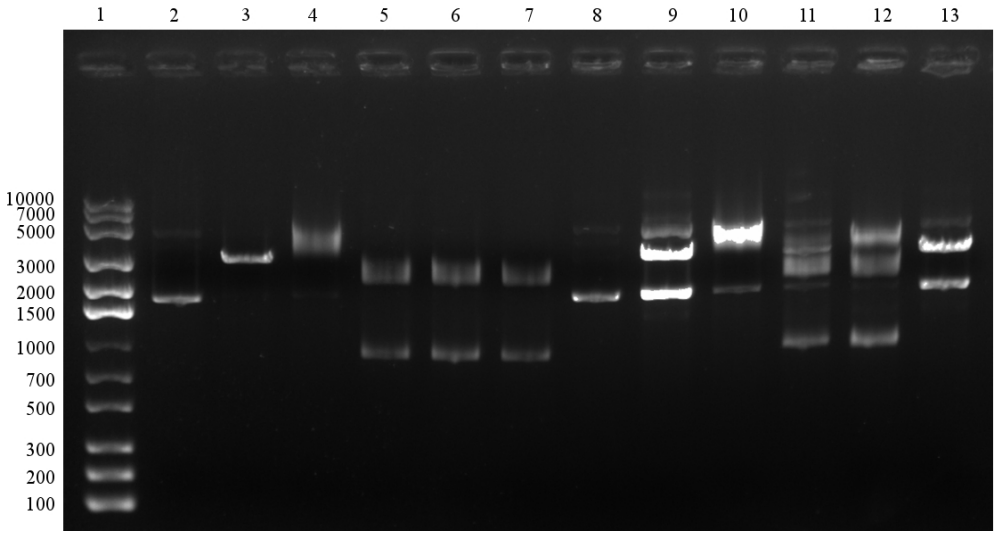

Figure 2: Gel electrophoresis 1, analysis of digestion products

Table 13: Gel Elecrophoresis 2

Well	Sample	Expected Size [bp]	Conclusion
1	DNA Ladder		
8 & 9 & 10	pSBIK3 dd	$\tilde{2}204$	Not assessable
11	T7 PCR undigested	346	Successful
12	T7 dd	170, 119 and 55	Likely successful*

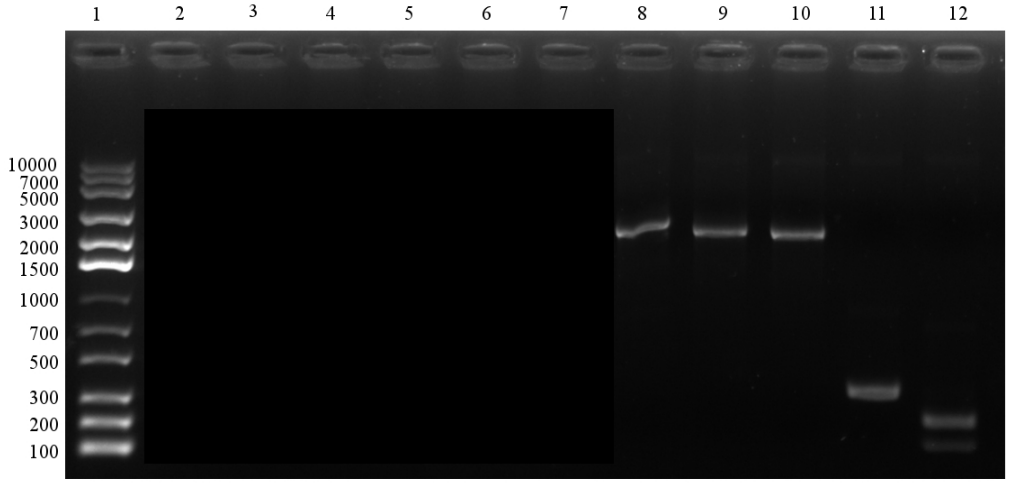

Figure 3: Gel electrophoresis 2, analysis of digestion products. Lanes 2-7 correspond to Lys samples, the analysis of which can be found in the lysostaphin lab book, week 3.

*Difficult to assess since T7 itself will likely not be visible on the gel. The two cut-off ends however, seem to be present, making successful digestion extremely likely.

To proceed, double digested EB, EC and pSB1K3 were gel purified. The resulting concentrations were as follows:
EB: 8.3 ng/μl
EC: 7.2 ng/μl
pSB1K3: 12.8 ng/μl

Discussion and Troubleshooting

In this experiment, it appeared as if PstI was consistently either not digesting as expected or the corresponding samples were running as smears on the gels. The smearing might be due to the Enzyme sticking to the DNA which can be abrogated by adding 1% SDS to the running buffer. Digestion of EB might have been successful whereas EC showed too many bands and bands of the wrong size. Lysostaphin showed no expected results at all. The following day, Ligation for the 3A assembly can be performed with the EB samples. EC can be attempted as well. As preparation however, T7 needs to be digested in sufficient quantities to perform ligation.

3 Ligation of pSB1K3-T7-EB and pSB1K3-T7-EC

Responsible

Aman Mebrahtu and Sigrun Stulz

Protocols used

- 3A Assembly - PCR (Taq)

Modifications and comments to protocols

At this point, all ligations are performed with a 1:3 vector:insert ratio

Experimental Set Up

pSB1K3 digested backbon	3.94 μl
Digested T7	0.58 μl
Digested EC	7.68 μl
OR	
Digested EB	6.73 μl
T4 DNA Ligase	0.5 μl
T4 DNA Ligase Buffer	0.2 μl
Total volume per reaction	20 μl

Results and Conclusions

Table 14: Gel Elecrophoresis of Ligation and Digestion Products

Well	Sample	Expected Size [bp]	Conclusion
1	DNA Ladder		
2	pSB1K3-T7-EB	2941	too little DNA loaded, inconclusive
3	pSB1K3-T7-EC	2975	too little DNA loaded, inconclusive
4	digested EB	857	weak band but appears successful
5	digested EC	891	weak band but appears successful

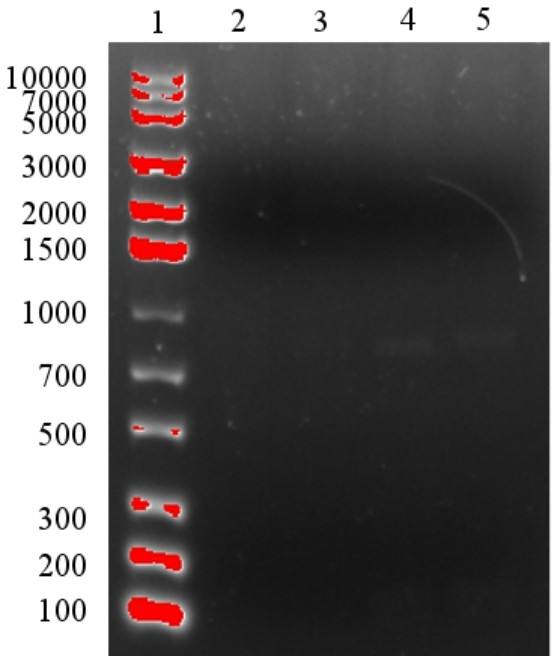

Figure 4: Gel electrophoresis of ligation products with inconclusive results

The results from running the ligation products on a gel were inconclusive because too little product was present, we hence tried to perform a PCR on the ligation product. The rationale was that if there was just a little product present, it should be amplified by the PCR. The primers should not be able to bind to digested biobricks and on a digested vector without insert, should only amplify small fragments between the binding site of the primers and the end of the digested vector.

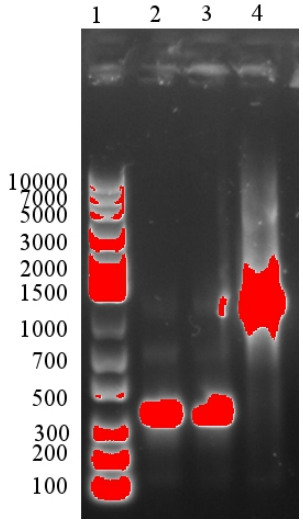

Figure 5: Gel electrophoresis of PCR on ligation products. Well 2 corresponding to PCR performed on pSB1K3-T7-EB and well 3 to pSB1K3-T7-EC. In well 3, the biobrick EB was loaded for size comparison. Bands in well 2 and 3 do not correspond to expected sizes.

Discussion and Troubleshooting

In this experiment, we performed a ligation with the goal of assembling a plasmid for the expression of EB and EC. Unfortunately we were unsure whether the ligation was successful since the 4 μl of ligation product that was loaded onto the gel was not sufficient to see clear bands and we were hesitant using more product because any subsequent transformation also requires a sizable amount of DNA. Therefore we attempted to perform a PCR on the ligation products with the aim of specifically amplifying the T7-EB fragment and confirm that ligation had in fact taken place. The fragments that were amplified by this reaction were not of the correct size though, and can not clearly be matched to any particular fragment we are using in our experiments.
Nevertheless, we decided to transform the ligation product since the fault might simply have been in loading too little DNA onto the gel.
In case the ligation would be re-done, using more DNA should be considered as well as perhaps varying the insert:vector ratio.

4 Transformation of pSB1K3-T7-EB and pSB1K3-T7-EC into TOP10

Responsible

Sigrun Stulz

Protocols used

- Transformation

Modifications to protocols

During recovery, cells were first incubated at 25°C on the shaker for 20min, due to technical difficulties. They were then moved to 37°C, 150rpm and left to recover for a full hour.

Experimental Set Up

Samples:
- pSB1K3-T7-EB
- pSB1K3-T7-EC
- RFP from competent cell test kit as positive control

Briefly, 5 μl ligation product or 3 μl RFP plasmid were transformed into 50 μl of KCM-competent TOP10 cells. Of the resulting cell suspension, 135 μl were streaked onto a kanamycin agar plate (chloramphenicol plate for the RFP) and 15 μl onto a antibiotic free plate to assess cell viability. All plates were incubated overnight at 37C.

Results and Conclusions

No Results at this stage, but colonies were observed after 18 h and in the following experiment 'Colony Picking' it became clear that the assembly might have been successful.

5 Colony Picking TOP10

Responsible

Sigrun Stulz

Protocols used

- Colony Picking - PCR

Modifications to protocols

To save reagents, each PCR reaction was performed in a total volume of 25 μl instead of 50 μl as proposed by manufacturer.

Experimental Set Up

Two colonies were observed for pSB1K3-T7-EB- and one for pSB1K3T7-EC whereas hundreds of colonies were seen for the RFP-transformed cells. All Eb and EC colonies were picked and colony PCR was performed according to protocol. Liquid cultures of a volume of 8 ml with kanamycin / chloramphenicol were grown in parallel to allow for making glycerol stocks and later on isolate plasmids.

Results and Conclusions

Well 1 DNA Ladder
Well 2 pSB1K3-T7-EB Colony 1
Well 3 pSB1K3-T7-EB- Colony 2
Well 3 pSB1K3-T7-EB- Colony 2
Well 4 pSB1K3-T7-EC

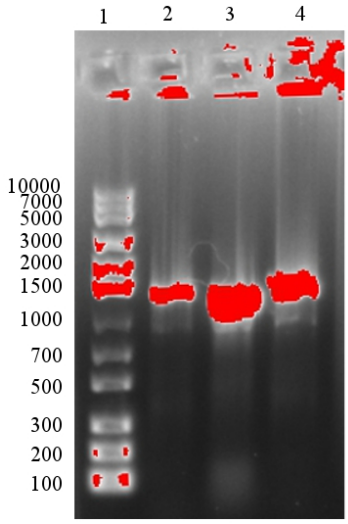

Figure 6: Colony PCR for pSB1K3-T7-EB and pSB1K3-T7-EC

Discussion and Troubleshooting

The gel for the colony PCR was quite smeary since 5 μl PCR product were loaded and the image was overexposed, leading to the red smears which indicate over-saturation at certain areas. However, bands of the right size can be observed and successful ligation is probable. The PCR product was kept for possible later sequencing.
Generally, a very low number of colonies was observed when transforming ligation products whereas the positive control worked fine. This likely indicates that the ligation product itself is not of a very high quality or quantity. For any following experiments, both digestion and ligation should be analysed carefully to make sure that high-quality ligation product can be transformed.

6 Glyercol Stocks and Plasmid Extraction of pSB1K3-T7-EB and pSB1K3-T7-EC

Responsible

Sigrun Stulz

Protocols used

- Glycerol Stocks
- Plasmid Extraction

Modifications and comments to protocols

Glycerol stocks are ideally created from exponential phase but were here for the purpose of easier timing made from overnight culture
Miniprep is usually made for a maximum of 5ml overnight culture but here all 8 μl were used for purification. All samples were spun down for 5 min at 4000 rpm and most of the supernatant removed. Cells were resuspended in remaining 1ml supernatant, transferred to Eppendorf tube and purification was begun.
During cell lysis step, lysis seemed incomplete and sample failed to clear up, likely because it contained too many cells. A second volume (250μl) of lysis buffer was added. Consequently, also twice the recommended amount of N-Buffer was added for neutralisation.

Results and Conclusions

After Miniprep, concentrations were measured:

pSB1K3-T7-EB Colony 1	214.52 μg/μl
pSB1K3-T7-EB Colony 2	193.39 μg/μl
pSB1K3-T7-EC	320.58 μg/μl
RFP	183.72 μg/μl

Discussion and Troubleshooting

Concentrations are high enough to continue to transform into BL21 cells.

7 Transformation of pSB1K3-T7-EB and pSB1K3-T7-EC into BL21

Responsible

Sigrun Stulz

Protocols used

- Transformation

Modifications and comments to protocols

Since plasmids are generally much easier to transform than ligation products, only 250ng were used for each transformation.

Experimental Set Up

Samples:
- pSB1K3-T7-EB Colony 1
- pSB1K3-T7-EB Colony 2
- pSB1K3-T7-EC
- RFP as positive control

For each plasmid, 250ng were transformed into 40 μll of KCM-competent BL21 cells.

Results and Conclusions

No results at this stage but the next day, hundreds of colonies were observed, indicating that transformation into BL21 seems to work well.

Discussion and Troubleshooting

Concentrations are high enough to continue to transform into BL21 cells.
In a next experiment, fewer cells should be plated or proper four-way streak should be performed to ensure that single colonies will be present for picking.